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**REVIEW ARTICLE**

# Central and peripheral autoantigen presentation in immune tolerance

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## SUMMARY

**Recent studies in both humans and experimental rodent models provide new insight into key mechanisms regulating tolerance to self-molecules. These recent advances are bringing about a paradigm shift in our views about tolerance to self-molecules with tissue-restricted expression. There is, indeed, mounting evidence that selected antigen-presenting cells (APCs) have the ability to synthesize and express self-molecules, and that such expression is critical for self-tolerance. Insulin is a key hormone produced exclusively by pancreatic β-cells and a critical autoantigen in type 1 diabetes. It provides an excellent example of a molecule with tissue-restricted expression that is expressed ectopically by APCs. The fact that APCs expressing insulin have been demonstrated in both thymus and peripheral lymphoid tissues suggests that they may play a role in insulin presentation in both the central and peripheral immune system. Experimental mice, in which insulin expression was altered, provide functional data that help to dissect the role of insulin presentation by APCs of the immune system. This review addresses recent literature and emerging concepts about the expression of self-molecules in the thymus and peripheral lymphoid tissues and its relation to self-tolerance.**


## INTRODUCTION

Immunological self-tolerance can be defined as a meta-stable state in which the immune system does not react destructively against self-molecules, cells or tissues. Lack or loss of self-tolerance is likely to result in autoimmune responses, cellular and tissue damage, and eventually the clinical onset of autoimmune disease. Interactions between antigen-presenting cells (APCs) and lymphocytes are critical for self-tolerance, and these are known to take place in both thymus (central tolerance) and peripheral lymphoid tissues (peripheral tolerance).[1,2] The mechanisms of positive and negative selection in the thymus are key to the shaping of a self-tolerant T-cell repertoire, especially in early life during the maturation of the immune system. In the thymus, developing lymphocytes with no marked reactivity against self-peptides are positively selected in the thymic cortex and enter the circulation as mature lymphocytes. In contrast, developing lymphocytes with marked reactivity against self-peptides undergo negative selection (deletion) in the thymic medulla.

However, thymic selection has been considered as an effective tolerogenic mechanism only for widely expressed self-molecules. This assumption was based on the consideration that proteins with tissue-restricted expression would not be available for presentation in the thymus. Thus, tolerance to such proteins could only be achieved through mechanisms of peripheral tolerance. Peripheral tolerance mechanisms are indeed operative in extrathymic lymphoid tissues and include deletion, anergy, ignorance and regulatory cells,[3] and contribute to maintaining autoreactive lymphocytes under tight control. An important corollary of the above theories is that the presentations of self-molecules that have a tissue-restricted pattern of expression would be mainly a peripheral event and would be dependent on the capture of such self-molecules by APCs, in particular immature dendritic cells (DCs).[4]

Recent evidence demonstrates that a large number of molecules, including many with tissue-restricted expression (or 'peripheral' proteins), are expressed in the thymus.[3,5,6] The expression of these proteins correlates, in most cases, with the presence of the corresponding transcript in the thymus. This discovery, together with studies demonstrating the expression of self-molecules also in peripheral lymphoid tissues, offers new insight into the role of self-antigen presentation in determining tolerance. Moreover, the ectopic transcription of genes coding for self-molecules with tissue-restricted expression suggests that capturing may not be the sole mechanism underlying the presentation of self-molecules. This review will focus on studies that have examined the role of insulin expression in lymphoid tissues as a prototypical example of a self-molecule with tissue-restricted expression for which novel findings in humans and animal models offer the opportunity to dissect the role of self-antigen presentation in regulating self-tolerance.

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## INSULIN AND OTHER SELF-MOLECULES WITH TISSUE-RESTRICTED EXPRESSION ARE PRODUCED THROUGH ECTOPIC GENE TRANSCRIPTION IN BOTH THYMUS AND PERIPHERAL LYMPHOID TISSUES

A review of the recent literature demonstrates that a large number of self-molecules with tissue-restricted or mainly peripheral expression are expressed in the thymus, at the mRNA and/or protein levels, in both humans and rodents.[5] These molecules include pancreatic and thyroid hormones, neuroendocrine molecules and many other proteins.[3,6–12] In some cases, these proteins are known target autoantigens in autoimmune disease. Proinsulin/insulin, glutamic acid decarboxylase [both 65 000 and 67 000 molecular weight isoforms (GAD65/67)] and a tyrosine-phosphatase-like protein known as IA-2, are targeted by islet-specific autoimmune responses in type 1 diabetes (T1D or IDDM); thyroid peroxidase and thyroglobulin are autoantigens in autoimmune thyroid diseases (AITD); autoimmune responses to myelin basic protein (MBP) and myelin proteolipid protein (PLP) are typically seen in multiple sclerosis (MS) in humans, as well as in the corresponding mouse model of experimental autoimmune encephalomyelitis (EAE).

Insulin gene transcription in the mouse thymus was originally reported by Jolicouer *et al*.[7] during fetal development and for a few weeks after birth; this was followed by similar reports for human[8,9] and rat[13] thymus. We surveyed human thymus specimens obtained from aborted fetuses, stillborn babies and children who underwent cardiac surgery (age-range: 20 weeks of fetal development to 13 years of age). The human insulin gene (*INS*) transcript was observed in almost all of the specimens tested.[8] Later studies demonstrated its expression, even in the thymus of a 56-year-old individual.[14] Other transcripts coding for T1D autoantigens (GAD, IA-2) were also detected.[8,14,15] Sospedra *et al*.[10] performed a more comprehensive survey of the human thymus, which included:

(1) Self-antigens with tissue-restricted expression but present in the circulation at high (albumin), intermediate (thyroglobulin), or low (insulin, glucagon) concentrations.
(2) Self-antigens with tissue-restricted expression that are not detectable in the circulation (thyroid peroxidase, GAD65/67).
(3) Classical sequestered antigens (MBP, the retinal S antigen).

Genes coding for these molecules were all found to be expressed in thymus specimens obtained from children between the ages of 8 days and 13 years. Another comprehensive survey of the mouse thymus provided further evidence that a variety of self-molecules, again including many autoantigens, are expressed in the thymus.[16] In this study, the ectopic transcription of several self-molecules was detected in the thymus of C57BL6 mice up to 34 weeks of age.[16]

We also found that transcripts for the T1D autoantigens insulin, GAD and IA-2, are present in human spleen and lymph nodes throughout life.[14,15] This indicates that the expression of genes coding for self-molecules is not limited to the thymus, but also takes place in peripheral lymphoid organs where it may contribute to tolerance. Similar results were obtained in the mouse (D. Hanahan *et al.*, personal communication). These findings may be critical for interpreting the results of some recent studies that are discussed below.

## DIFFERENTIAL EXPRESSION OF SELF-MOLECULES AND AUTOIMMUNITY

It may seem paradoxical that autoimmune responses could be observed against self-molecules if these were expressed in the thymus. However, several mechanisms controlling gene expression can influence the quantity and quality of expression of self-molecules in the thymus. Quantitative and qualitative differences in the expression of self-molecules may, in turn, affect the thymic selection and other mechanisms that require self-antigen presentation for achieving and maintaining self-tolerance. The best-characterized examples involve the genes coding for insulin, the expression of which is affected by allelic variation and imprinting in the human thymus,[8,9,17] and the islet molecule IA-2,[15] which, similarly to the MS autoantigen PLP, is subject to alternative splicing.[18,19] These and other genetic effects were collectively examined in a recent review.[5] The present article focuses on the regulation of insulin expression in humans and in mouse models in an effort to help to understand the role of its expression in the thymus and peripheral lymphoid tissues.

### Regulation of *INS* transcription in the human thymus

The regulation of *INS* transcription in the human thymus provides an example of how levels of self-antigen expression may affect tolerance and susceptibility to autoimmune responses against a specific molecule. The levels of insulin mRNA expression in the human thymus correlate with allelic variation at the *IDDM2* susceptibility locus. This locus corresponds to a polymorphic variable nucleotide tandem repeat (VNTR) sequence,[20–24] which mediates a transcriptional signal in the steady state.[25] The transcriptional activity in the thymus was found to be ≈200–300% higher for *INS* transcripts encoded by *IDDM2* alleles that were clinically associated with resistance to the development of diabetes, and conversely it was lower for transcripts encoded by diabetes-predisposing *IDDM2* alleles.[8,9] Furthermore, there is evidence that parent-of-origin effects may affect the diabetes susceptibility associated with the *IDDM2* locus.[22–24,26–28] These parent-of-origin effects may be mediated by parental imprinting,[29] a mechanism that could suppress the expression of one of the two insulin genes in both thymus and peripheral lymphoid organs. There is indeed evidence that monoallelic expression of the insulin gene takes place in the thymus,[8,9] the spleen[30] and in the yolk sac.[31] Remarkably, the non-expressed allele in thymus and spleen was always the one *in cis* with the protective *IDDM2* alleles. This suggests that imprinting may prevent the protective effect by silencing transcription from these alleles and, in turn, dramatically reduce insulin production in the thymus. Overall, the risk of diabetes that is determined by allelic variation and parent-of-origin effects at the *IDDM2* locus appears to be dependent on the levels of *INS* transcription in the thymus. Differential transcriptional regulation may determine the amounts of insulin produced and, in turn, influence the efficiency of thymic selection processes involving insulin-specific autoreactive T cells. The increased transcription levels detected in thymus fit well with the dominant protective effect associated with certain *IDDM2* alleles, as higher insulin levels in the thymus may more efficiently induce the negative selection of insulin-specific T

lymphocytes (or improved selection of regulatory T cells). In contrast, homozygosity for diabetes-predisposing alleles results in lower insulin levels, which may be associated with a less efficient deletion of insulin-specific autoreactive T cells (or impaired selection of regulatory T cells). *INS* transcription in the human thymus also correlates with protein production,[8,9] where proinsulin appears to be the main gene product.[14] Thymus cells expressing proinsulin are not likely to possess the refined machinery necessary to process proinsulin to mature insulin. Rather, it would seem that proinsulin may be processed into peptides for presentation to developing lymphocytes. Proinsulin expression may be sufficient to obtain tolerance to insulin because most of the known immunodominant epitopes identified as targets of the insulin autoimmune responses in type 1 diabetes are shared by both insulin and proinsulin.[32–36]

### Animal models of insulin expression in the thymus

Besides the work, described above, carried out in humans, and earlier studies demonstrating the generic effects of antigen levels on thymic selection *in vitro*,[1,37–40] studies in transgenic or knockout mice have provided more direct evidence that insulin thymic levels can dramatically affect the development of self-tolerance to insulin. The mouse models studied thus far most often involve manipulations of the non-obese diabetic (NOD) mouse, a spontaneous model of autoimmune diabetes that closely resembles the human disease.[41,42] NOD mice develop a lymphocytic infiltrate of the pancreatic islets (insulitis), which leads to β-cell destruction and the development of diabetes in ≈80% of the female and 35–40% of the male mice by 30 weeks of age. The target autoantigens of the autoimmune process in mice overlap with those in humans, as they include proinsulin/insulin, GAD and IA-2. Autoimmune responses to insulin and proinsulin are known to be among the earlier ones to develop during the natural history of the disease,[43–50] and both humoral and cellular responses are known to play a key role in diabetes development. In particular, immune responses against a proinsulin epitope encompassing the B chain/C-peptide junction (B24-C33, B24-C36) and a B-chain epitope encompassing the B9-23 residues (B9-23 for CD4 T cells, B15-23 for CD8 T cells) have been associated with diabetes in mice and humans.[35,36,44,48,51–54] Responses to other epitopes of proinsulin/insulin have also been reported in patients and prediabetic subjects (C47-A66, C37-A66, C56-A72, B11-C41, B11-27).[49,50,55] As with humans, the major histocompatibility complex (MHC) I-A locus, corresponding to the human leucocyte antigen (HLA)-DQ, provides the largest genetic contribution to disease susceptibility.[56,57]

Unlike humans, however, mice have two non-allelic insulin genes, which are located on two different chromosomes, *Ins1* and *Ins2*.[58] They are both expressed in pancreatic β-cells, although they encode slightly different proteins (Table 1). Moreover, allelic variation is not observed in the highly inbred NOD mice, and VNTRs are not known to exist in mice. Chentoufi *et al.*[59] have recently established a very elegant mouse model in which mice lack the expression of either one or two copies of the two insulin genes. While insulin production in pancreatic β-cells is largely unaffected as the result of compensatory mechanisms,[59,60] thymic expression is dependent on the number of gene copies present. These studies established that of the two insulin genes, *Ins2* is the one that is predominantly, if not almost exclusively, expressed in the thymus. This model of graded insulin deficiency in the thymus also showed a linear correlation with insulin gene copy numbers. Mice expressing low thymic insulin levels presented spontaneous peripheral reactivity to insulin and the C-peptide, whereas mice with normal levels showed no significant response.[59] Thus, this work provides functional evidence that thymic insulin levels play a key role in the selection of insulin-specific T cells and supports the concept that variation in *INS* levels, determined by allelic variation and imprinting phenomena at the *IDDM2* locus, is the mechanism by which this locus influences disease risk. Of note, imprinting phenomena affect the expression of both *Ins1* ands *Ins2* in the yolk sac,[61] although it remains to be established whether this phenomenon also occurs in the mouse thymus.

**Table 1.** Amino acid sequences encoded by the mouse *Ins2* and *Ins1* genes

| | |
|---|---|
| 1–24 Signal peptide | |
| ($n$ = 6; residues 4, 5, 6, 15, 19, 20) | |
| *Ins2* | MALWMRFLPLLALLFLWESHPTQA |
| *Ins1* | MALLVHFLPLLALLALWEPKPTQA |
| 25–54 B chain | |
| ($n$ = 3; residues 33, 37, 53) | |
| *Ins2* | FVKQHLCGSHLVEALYLVCGERGFFYTPMS |
| *Ins1* | FVKQHLCGPHLVKALYLVCGERGFFYTPKS |
| 55–56 | |
| *Ins2* | RR |
| *Ins1* | RR |
| 57–87 C-peptide | |
| ($n$ = 5; residues 64, 71, 74, 75, 86) | |
| *Ins2* | EVEDPQVAQLELGGGPGAGDLQTLALEVAQQ |
| *Ins1* | EVEDPQVEQLELGGSPG**DLQTLALEVARQ |
| 88–89 | |
| *Ins2* | KR |
| *Ins1* | KR |
| 90–110 A-chain | |
| *Ins2* | GIVDQCCTSICSLYQLENYCN |
| *Ins1* | GIVDQCCTSICSLYQLENYCN |

Amino acid sequences encoded by the ***Ins2*** (top) and ***Ins1*** (bottom) genes are shown.[58] Amino acid residues that differ between the two sequences are underlined.

Another important line of support for the hypothesis that levels of insulin expression in the thymus are crucial for thymic selection and diabetes susceptibility came from studies that utilized NOD mice overexpressing the *Ins2* gene under the MHC class II promoter.[62] The increased levels of *Ins2* expression in the thymus of the transgenic mice compared with non-transgenic NOD mice was associated with the complete prevention of insulitis and diabetes. These results are consistent with the notion that increased levels of insulin expression in the thymus could result in a more efficient deletion of insulin-specific T cells in the thymus of NOD mice. The complete prevention of insulitis in this model also suggests that insulin may play a critical role as an early autoantigen.

Using a contrasting approach, Thebault-Baumont *et al.*[63] bred mice with a null *Ins2* mutation[64] onto the NOD

background, so that most of the known susceptibility loci (including the major predisposing MHC locus, I-Ag7) were present in the fourth backcross generation. NOD *Ins2*$^{-/-}$ mice develop accelerated insulitis and diabetes, and similar results were obtained by Moriyama *et al*.[65] Thus, two independent studies involving NOD *Ins2*$^{-/-}$ mice show that essentially all mice develop diabetes, regardless of gender, while it is known that male NOD mice are less prone to diabetes development.[63,65] Moreover, *Ins2*$^{-/-}$ NOD mice display increased immune responses to insulin (autoantibodies), and splenocytes from these mice have an increased capacity to transfer diabetes compared with splenocytes from 'wild-type' NOD females.[63] In contrast, NOD mice lacking expression of the *Ins1* gene are protected from the development of diabetes.[65] Diabetes and insulitis are markedly reduced in *Ins1*$^{-/-}$ mice, with virtually no mice developing insulitis and diabetes. Heterozygous mice also show a decreased incidence of diabetes as well as a delayed appearance of symptoms. However, *Ins1*$^{-/-}$ NOD female mice express insulin autoantibodies at levels similar to those produced by the wild-type NOD female mice, suggesting that the production of insulin antibodies may not be controlled by the expression of insulin in the thymus.

Based on the studies of Chentoufi *et al*.,[59] and the above findings in *Ins2*[63,65] and *Ins1*[65] knockout mice, one could postulate that the protective effect afforded by the lack of the *Ins1* gene product reflects differences between the two insulin proteins produced by the mouse. Moreover, differences in the expression pattern between the thymus and pancreas may be critical for diabetes pathogenesis. Because *Ins2* is normally the only insulin gene actively expressed in the thymus, NOD *Ins2*$^{-/-}$ mice would display accelerated diabetes because they would not have any insulin in the thymus that could be used to delete insulin-specific T cells. On the other hand, NOD mice normally develop diabetes and autoimmune responses to proinsulin/insulin epitopes, despite the expression of *Ins2* in the thymus. This suggests that NOD mice may express insufficient amounts of *Ins2* in the thymus and, consistent with this assumption, one report has shown that NOD mice express ≈50% less insulin in the thymus compared with BALB/c mice.[66] However, qualitative differences between Ins2, which is made in the thymus, and Ins1, which is made in the pancreas but not in the thymus, could also be critical for diabetes development. The studies of Moriyama *et al*.,[65] demonstrating protection from diabetes in *Ins1* NOD knockout mice, support this hypothesis. In other words, the immune system may not be fully tolerant to *Ins1* because the thymus expresses only *Ins2*. Thus, lack of *Ins2* expression completely abolishes tolerance to both insulin variants and accelerates diabetes because *Ins1* is still expressed by the pancreas. Lack of *Ins1* expression only affects expression in the pancreas, and once *Ins1* is not expressed in the pancreas, diabetes does not develop because mice should be tolerant to *Ins2* as this is expressed in the thymus. Thus, it would appear that NOD mice are essentially tolerant to *Ins2*, but are not tolerant to *Ins1*. These concepts are illustrated in Fig. 1.

Tables 1 and 2 show the sequences of the two proteins and highlight the differences existing between them. It can be noted that with the exception of one epitope encompassing the A chain, the shown target epitopes have sequence differences between *Ins2* and *Ins1*, which may favour the onset of autoimmune responses. Further experiments by Thebault-Baumont *et al*.[63] support this interpretation. They immunized NOD *Ins2*$^{-/-}$ mice and wild-type NOD mice with a series of *Ins2* peptides. Both mouse strains mice showed significant

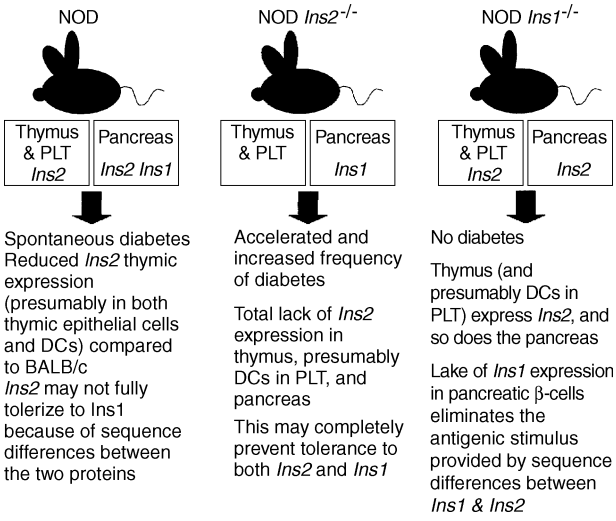


**Figure 1.** Illustration of the relationship between the expression of insulin in the thymus and peripheral lymphoid tissues (PLT), mediated by thymic epithelial cells and bone marrow-derived dendritic cells (DCs), and diabetes development in the mouse strains. The figure also highlights the different outcomes associated with the manipulation of the *Ins2* and *Ins1* genes in relation to their expression in the thymus and PLT, as well as in the pancreas. Please see the text for details. NOD, non-obese diabetic.

**Table 2.** Known proinsulin/insulin target epitopes in autoimmune diabetes

| | |
|---|---|
| Epitope 48–62 | |
| (B chain/C-peptide junction, aa B24-C36) | |
| *Ins2* | FFYTPMSEVEDPQ |
| *Ins1* | FFYTPKSEVEDPQ |
| Epitope 33–47 | |
| (B chain aa 9–23, also includes epitope 15–23) | |
| *Ins2* | SHLVEALYLVCGERG |
| *Ins1* | PHLVKALYLVCGERG |
| Epitope 14–30 | |
| (signal peptide aa 14–20, B chain aa 1–6) | |
| *Ins2* | LLFLWESHPTQAFVKQHL |
| *Ins1* | LLALWEPKPTQAFVKQHL |
| Epitope 20–35 | |
| (signal peptide aa 20, B chain aa 1–11) | |
| *Ins2* | HPTQAFVKQHLCGSHL |
| *Ins*1 | KPTQAFVKQHLCGPHL |
| Epitope 71–88 | |
| (C-peptide) | |
| *Ins2* | GPGAGDLQTLALEVAQQK |
| *Ins1* | SPG**DLQTLALEVARQK |
| Epitope 88–103 | |
| (A chain) | |
| *Ins2* | KRGIVDQCCTSICSLY |
| *Ins1* | KRGIVDQCCTSICSLY |

The table shows proinsulin/insulin target epitopes reported by several authors.[35,36,44,52–54,63,67] Amino acid (aa) residues that differ between the two sequences are underlined.

T-cell responses to immunization with the *Ins2* peptides 14–30, 20–35, 33–47 and 71–88 (Tables 1 and 2). This would seem logical, as both strains are known to have impaired tolerance to proinsulin/insulin, and immunization with *Ins2* peptides will probably induce cross-reactive responses to *Ins1*. However, NOD *Ins2*$^{-/-}$ mice showed a significant interleukin-2 (IL-2) response against the *Ins2* peptide 88–103, a response that is not seen in the 'wild-type' NOD strain[67] and that targets a region of complete homology between the A chains of the two mouse insulin proteins. The fact that the lack of *Ins2* expression in the thymus is associated with the ability to respond to an additional epitope not normally targeted by NOD mice supports the interpretation presented above. In fact, *Ins2* knockout mice would only express *Ins1* in the pancreas and would have no ability to tolerize to *Ins1* in the thymus; hence, a response against a region that is identical between the two proteins can develop. In contrast, 'wild-type' NOD mice express *Ins2* in the thymus and do not break tolerance to this epitope, consistent with the expectation that they would be tolerant to a region of the *Ins1* protein that is identical to that of the *Ins2* expressed in the thymus.

## CELLS EXPRESSING SELF-MOLECULES IN THYMUS AND PERIPHERAL LYMPHOID TISSUES

The evidence discussed so far suggests that the expression of self-molecules in the thymus leads to the deletion of autoreactive T cells and that quantitative and qualitative differences in the expression of self-molecules play a key role in this process. Deletion of autoreactive T cells has been observed both in thymus and peripheral lymphoid tissues.[1,68–70] Deletion is mediated by apoptosis and involves those lymphocytes recognizing a self-peptide expressed by a self-MHC molecule on the surface of an APC. Presentation of self-molecules in the thymus may also promote the positive selection of regulatory cells.[71,72] Thus, APCs expressing self-molecules may play a key role in inducing immunological tolerance to self-antigens. APCs in thymus and peripheral lymphoid tissues include bone marrow-derived DCs and macrophages, while thymic epithelial cells, including nurse cells, are found only in the thymus. Besides medullar thymic epithelial cells, cortical thymic epithelial cells and nurse cells may also play a role in negative selection.[11] Defining the phenotype of the cells expressing self-molecules in the thymus should facilitate their functional characterization, and a number of studies have investigated the nature of these cells.

Initial studies showed that the mouse thymus contains specialized cells expressing peripheral antigens such as insulin and other pancreatic molecules.[12] These cells were termed peripheral antigen expressing (PAE) cells.[6,12] Thymus transplants involving transgenic mice expressing the Tag antigen (SV40 T antigen) under the rat insulin promoter (RIP-Tag) provided functional evidence that PAE cells can mediate tolerance to the antigens they express. In these experiments, transplanting the thymus of RIP-Tag mice in non-transgenic mice resulted in central tolerance to the Tag antigen.[6,12]

Later studies by Throsby *et al.*[73] showed that PAE cells expressing insulin and other pancreatic hormones (glucagon, somatostatin, pancreatic polypeptide) in the C57BL6 thymus

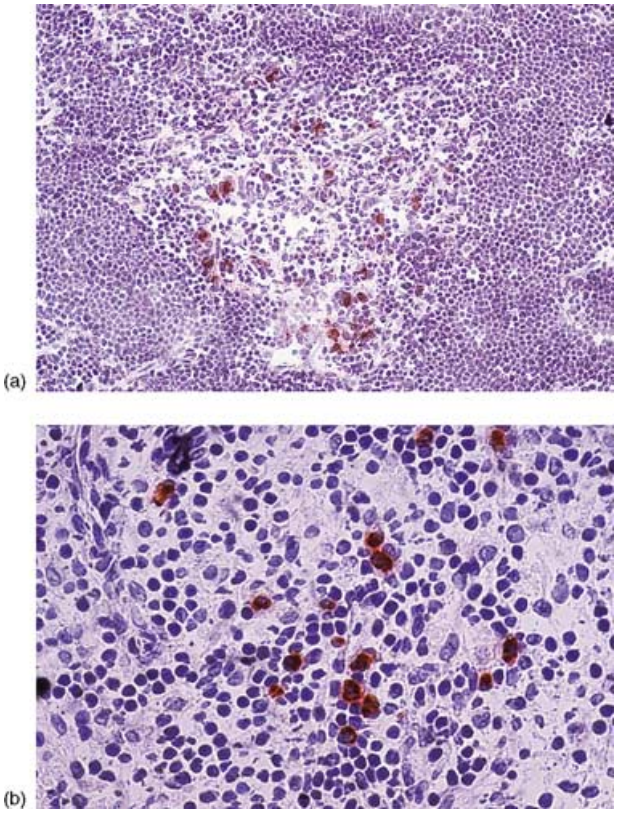


**Figure 2.** (a) Proinsulin-positive cells in human thymus, stained on a frozen section using streptavidin–biotin–peroxidase and the AEC substrate (red), counterstained with haematoxylin (×64 magnification). (b) Proinsulin-positive cells in human spleen, stained on a frozen section using streptavidin–biotin–peroxidase and the AEC substrate (red), counterstained with haematoxylin (×128 magnification).

belong to the DC and macrophage lineages. We studied human thymus sections from aborted fetuses, stillborn babies, children who underwent cardiac surgery, or adult organ donors (age-range: 20 weeks of fetal development to 56 years of age).[14] Sections were stained using several antibodies to T1D autoantigens, including insulin, GAD and IA-2. We found sparse cells, mostly localized in the thymic medulla or at the cortico-medullary junction, which stained positive for proinsulin (Fig. 2), GAD and IA-2. Using double immunofluorescence, we demonstrated that the same cells expressing proinsulin could co-express GAD and IA-2.[14] These cells also co-stained for CD11c, CD83, CD40, CD14, CD80, CD86, CD8α and HLA class II. Among APCs, DCs express CD11c and CD83, while macrophages typically express CD14. These findings indicate that thymic cells expressing islet cell antigens bear surface markers of bone marrow-derived DCs and macrophages. Similarly to the work of Throsby *et al.*,[73] in C57BL6 mice, we were unable to observe co-localization of any of the T1D autoantigens above with cytokeratin, a marker of thymic epithelial cells.[14] Thus, studies in both human and mouse thymus provided similar results, even if different methodological approaches were used [double immunofluorescence in tissue sections from human thymus and fluorescence-activated cell

sorter (FACS)/reverse transcription–polymerase chain reaction (RT–PCR) in cell fractions isolated from mouse thymus]. We have since confirmed this phenotypic characterization using flow cytometry.[74]

Given the phenotype established by these studies, it should be no surprise that insulin, GAD and IA-2 transcripts, as well as cells expressing these molecules, were also found in human peripheral lymphoid tissues, such as spleen (Fig. 2) and lymph nodes.[14] These cells expressed the same phenotypic markers of their thymus counterparts, suggesting that bone marrow-derived APCs can express T1D autoantigens also in peripheral lymphoid organs. Another consistent feature of these cells in both thymus and spleen was the formation of 'rosettes' in which lymphocytes surrounded proinsulin-positive cells. Both the localization within the thymic medulla and the rosettes are typical of DCs and, to a lesser extent, of macrophages.[75] Negative selection is compartmentalized in the thymic medulla and is mostly mediated by DCs,[76] and our findings suggest that self-APCs include DCs that may be involved in the clonal deletion of self-reactive lymphocytes. This hypothesis is strengthened by the observation, in both human thymus and spleen, of rosettes consisting of proinsulin-positive cells in intimate contact with apoptotic cells.[14] This is consistent with reports that regulatory DCs kill T cells by inducing apoptosis[1] and that T-cell apoptosis also takes place in the spleen.[68–70] DCs are known to be capable of stimulating the immune response or mediating tolerance, depending on their maturation status and phenotype.[4,77] Thymic expression of self-molecules can provide a strong tolerogenic signal, but this signal may be incomplete. Although tolerance may be initiated in the thymus, peripheral mechanisms may be necessary to complete the tolerogenic process or, more likely, to provide the continuous exposure to self that is necessary to control emerging cells with autoreactive potential.[4]

Other investigators, however, produced evidence that thymic epithelial cells express self-molecules in the thymus, including insulin and other T1D autoantigens. Sospedra *et al.*[10] used RT–PCR to detect the presence of transcripts encoding several self-molecules in human thymic cell fractions, and found transcripts of several autoantigens in both fractions enriched for either DCs or thymic epithelial cells. Recent studies by Derbinski *et al.*,[16] in the thymus of C57BL6 mice, demonstrated that medullary thymic epithelial cells express self-molecules with tissue-restricted expression. These authors detected the transcripts of a number of self-molecules, including insulin, GAD, IA-2, MBP, PLP, thyroglobulin, etc., in sorted populations of medullar thymic epithelial cells but not in cortical epithelial cells, DCs and macrophages.

Thus, there is evidence that both bone marrow-derived APCs and thymic epithelial cells express self-antigens with tissue-restricted expression in the thymus. However, the relative importance of self-molecule expression by bone marrow-derived APCs and thymic epithelial cells is not completely defined. PAE cells with a DC phenotype exist not only in human thymus but also in peripheral lymphoid tissues, such as spleen and lymph nodes.[14] These tissues, similarly to the thymus, also expressed transcripts for self-molecules such as the T1D autoantigens insulin, GAD and IA-2.[14,15] These findings support the concept that DCs are involved in the production of self-molecules because thymic epithelial cells do not exist in peripheral lymphoid tissues. Given the phenotypic identity of cells expressing proinsulin and other T1D autoantigens in the human thymus and peripheral lymphoid tissues, it would seem reasonable that bone marrow-derived APCs would be able to transcribe genes coding for self-molecules. Consistent with this interpretation, we have detected insulin mRNA from CD11c-positive DCs after sorting.[78] Functional evidence for a tolerogenic function of bone marrow-derived APCs derives from the studies of Steptoe *et al.*[79] who demonstrated that syngeneic transplantation of haematopoietic stem cells encoding *Ins2* transgenically targeted to APCs totally prevents the development of spontaneous autoimmune diabetes in NOD mice. Thus, bone marrow-derived APCs expressing self-molecules have tolerogenic function in a stringent autoimmune model of diabetes. Recent studies have also investigated the role of the autoimmune regulator protein, encoded by the *AIRE* gene, in determining susceptibility to the autoimmune polyendocrinopathy candidiasis ectodermal distrophy (APECED) syndrome.[80] The AIRE protein is a transcription factor that is primarily expressed in thymic medullary epithelial cells and monocyte DCs in the thymus, but also in a rare subset of cells in the lymph nodes, spleen and fetal liver.[81] The APECED syndrome is characterized by the presence of autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy. Variable combinations of autoimmune endocrine diseases such as Addison's disease, hypoparathyroidism and type 1 diabetes are often seen in patients with APECED.[82] The APECED syndrome is caused by mutations in the AIRE gene.[83] Two lines of AIRE knockout mice have been generated in order to dissect the role of AIRE in the autoimmune manifestations of the APECED syndrome.[84,85] These mice develop normally, have a normal distribution of B and T cells, normal thymic maturation and T-cell activation. However, the T-cell receptor (TCR)-Vβ repertoire is altered in peripheral T cells and, when mice are immunized, the peripheral T cells have a three- to fivefold increased proliferation, suggesting a role for AIRE in maintaining homeostatic regulation of the immune system.[84] These mice also display variable degrees of the autoimmune features of APECED, including multiorgan lymphocytic infiltration, circulating autoantibodies and infertility.[84,85] Another striking feature observed in the AIRE knockout mice is the lack of expression of a variety of genes coding for self-molecules, including the insulin gene, in thymic epithelial cells.[85] While more studies are needed to assess the consequences of the lack of self-antigen expression by thymic epithelial cells in AIRE-deficient mice, these findings collectively underscore the importance of both DCs and thymic epithelial cells in expressing self-molecules and mediating tolerogenic signals.

## CONCLUSIONS

The research discussed here provides evidence that self-molecules with tissue-restricted expression are expressed in the thymus, where such expression may promote the development of self-tolerance. Genetic factors modulate the expression of specific genes and, in turn, influence susceptibility to autoimmune responses against the molecules encoded by these genes. In addition, some of the self-molecules studied, and in particular islet cell molecules that are targets of autoimmunity

in T1D, are also expressed in peripheral lymphoid tissues. Transgenic and knockout mouse models, in which the expression of the insulin genes is altered, exemplify the tolerogenic role of self-antigen expression in the thymus and peripheral lymphoid tissues. These models also help in understanding the mechanisms underlying insulin autoimmune responses. Progress has been made in the characterization of the cells expressing self-molecules in the thymus. There is evidence that both bone marrow-derived APCs and thymic epithelial cells express self-molecules. Unlike thymic epithelial cells, bone marrow-derived APCs are abundantly represented in both thymus and peripheral lymphoid tissues, and may also contribute to self-tolerance in the periphery. The existence of cells, capable of expressing self-molecules, in both the central and peripheral compartments of the immune system, suggests that both thymic and peripheral tolerance mechanisms are likely to be affected by the ectopic expression of self-molecules in DCs and thymic epithelial cells. Given the property of DCs to autonomously express self-antigens, the expression of self-antigens by APC does not seem to be entirely dependent on antigen capture. Further studies should address whether there are differences between naturally expressed, processed and presented epitopes and those that are captured and then presented, and whether these differ in their ability to stimulate or inhibit the immune response. Most studies addressing responses to specific epitopes have used synthetic peptides; only a few have studied naturally processed and presented epitopes.[86] Essentially no studies have yet addressed responses to naturally expressed, processed and presented epitopes, and it is plausible that the epitopes which are expressed naturally by APCs, either by autonomous production or capture, may be more likely to influence tolerance or immune responses. It also remains to be tested whether the expression of self-antigens by DCs in the peripheral immune system can only induce tolerance or, under particular conditions, result in the activation of immune responses. Further investigations are required to fully establish the phenotype and function of APCs expressing self-molecules, the nature of the epitopes expressed, and how these factors influence the immune system responsiveness towards self.

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